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Use It or Lose It? Loss of Grazing Defenses during Laboratory Culture of the Digestion-Resistant Green Alga Oocystis

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Use it or lose it? Loss of grazing defenses during laboratory culture of the digestion-resistant green alga *Oocystis*

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A strain of the green alga *Oocystis* isolated from a lake showed a decline in digestion defenses and an increase in growth rate over 3 years of culture. Changes over time were corroborated by comparisons between a sub-strain grown continuously in light and two sub-strains kept refrigerated in the dark most of the time. Continuous culture in light leads to a sharper decline in grazer defenses and an increase in growth rate. The decline in digestive defenses was evidenced by increases in carbon assimilation efficiency and juvenile growth rate of *Daphnia* feeding on the alga as well as decreases in the incidence and thickness of the protective gelatinous sheath of the alga. Moreover, two strains of *Oocystis* from culture collections isolated decades earlier showed no evidence of grazer defenses in comparison with a high quality control alga. At high algal concentrations, *Daphnia* juvenile growth rate increased from 0.10 to 0.38 day^{-1} for field-isolated *Oocystis* over time in culture and ranged from 0.57 to 0.61 day^{-1} for the three undefended algae. Our experiments suggest that grazing favors the evolution of digestion defenses in *Oocystis* at the cost of slower growth and show that caution is needed when using cultured algae in food chain experiments.

KEYWORDS: phytoplankton evolution; cost of defense; defense versus growth trade-off; *Daphnia*

INTRODUCTION

Phytoplankton show a variety of defenses against grazers, including morphologies (size and shape) that limit ingestion, viable gut passage and toxic or distasteful chemicals (reviewed by DeMott, 1989; Sterner, 1989). A general component of defense theory is that defenses are costly and there should be an evolutionary trade-off between defense and growth. In support of this trade-off hypothesis, a wide variety of defended freshwater algae show lower growth rates in comparison with poorly defended

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taxa (Agrawal, 1998). However, because algal growth rates are strongly influenced by phylogeny, cell size and other factors (Edwards *et al.*, 2012), strong evidence for the evolution of defense-growth trade-offs in phytoplankton should come from comparisons between intraspecific clones and closely related species (Yoshida *et al.*, 2004).

Natural phytoplankton populations show genetic (clonal) variation in growth rate (Brand, 1981, 1989; Rynearson and Armbrust, 2000; Wilson *et al.*, 2006), morphological defenses (Vanormelingen *et al.*, 2009), colony size (Wilson *et al.*, 2006), palatability (White *et al.*, 2011) and chemical defenses (Wilson *et al.*, 2006). Yet, there has been very limited testing for defense-growth trade-offs between clones isolated from field populations. In one example, a poorly grazed strain of the coccolithophore *Emiliania huxleyi* from a tropical ocean showed lower ammonium uptake and lower growth than two readily grazed strains of the same species from temperate coastal waters (Sunda and Hardison, 2010).

Phytoplankton clonal isolates show evolutionary changes as they accumulate mutations and adapt to culture conditions (reviewed by Lakeman *et al.*, 2009). Surprisingly, populations initiated as clonal isolates have been shown to develop cryptic genetic variation in sensitivity to heavy metals (Lakeman and Cattolico, 2007), competitive ability (Costas *et al.*, 1998) and growth rate and grazer defenses (Yoshida *et al.*, 2004; Becks *et al.*, 2010). If not taken into account, such evolutionary change and genetic diversity within what were originally clonal isolates potentially complicate the interpretation of laboratory experiments. However, experiments with clones isolated from culture collection strains have also provided important insights in the interplay between grazer-phytoplankton population dynamics and algal evolution (Yoshida *et al.*, 2003; Meyer *et al.*, 2006; Becks *et al.*, 2010).

The rate of evolution in clonal cultures appears to be highly variable, presumably depending on culture conditions, population size, generation time and the traits being measured (Lakeman *et al.*, 2009). Theoretical modeling predicts initially slow changes in a clonal, asexual population, as mutations gradually accumulate, followed by more rapid evolution within a few hundred days (Lynch *et al.*, 1991). Short generation times and large population sizes in algal cultures favor evolutionary change, but the timing of specific phenotypic changes is difficult to predict. For example, one subculture of a dinoflagellate stopped producing saxitoxin ~40 years after isolation while another subculture kept under similar conditions continued to produce the toxin (Martins *et al.*, 2004). The loss of toxin production during long-term culture has also been reported for cyanobacteria (Kaebernick *et al.*, 2001; Schatz *et al.*, 2005). At the other time extreme, Costas *et al.* (1998) showed that clonal isolates of a dinoflagellate evolved improved interclonal competitive ability during experiments lasting only 5 weeks.

Since algal cultures are maintained in the absence of grazers, we can predict the relaxation of selection for grazer defenses during laboratory culture. The rate at which grazer defenses are lost should depend, in part, on the cost of defense under the poorly characterized selection pressures of batch culture. Nevertheless, *Chlorella* strains showed substantial interclonal variation in defenses against rotifer predation that traded off with competitive ability (Yoshida *et al.*, 2004) and growth rate (Meyer *et al.*, 2006) more than 50 years after the clonal isolates were deposited into a culture collection. In these examples, variation along a defense versus growth gradient apparently evolved and was maintained in the complete absence of grazer mortality. Comparisons between defended and undefended strains of the coccolithophore *E. huxleyi* were conducted ~10–40 years after the strains were isolated from the field (Sunda and Hardison, 2010). Thus, grazer defenses were apparently maintained over thousands of generations in the absence of grazing mortality. Moreover, we do not know how closely the strains used in the experiments reflected the traits of their field-isolated ancestors. Thus, the few relevant studies provide limited evidence that grazer defenses are lost when phytoplankton are cultured in the absence of grazers.

Gelatinous algae, including many green algae and cyanobacteria, are covered by mucilaginous sheaths that can increase the chance of viable gut passage (Porter, 1973, 1975). However, digestion defenses are not the only potential adaptive advantage of such mucilaginous sheaths (reviewed by Reynolds, 2007). A gelatinous covering decreases specific gravity and may also increase colony size above the upper size limit for ingestion (e.g. Kampe *et al.*, 2007). Field experiments show that digestion resistance reduces food quality and that the relative abundance of algae with gelatinous sheaths often increases with grazing pressure (e.g. Porter, 1973; Vanni, 1987; Kerfoot *et al.*, 1988). Comparisons between Michigan lakes that differ in grazing pressure show that digestibility accounts for most of the variation in *Daphnia* growth rate and food quality (DeMott and Tessier, 2002; Tessier and Woodruff, 2002). A trade-off between defense and growth rate might explain the reduced success of digestion resistant, gelatinous algae when grazing is weak.

In laboratory mesocosm experiments, DeMott and Van Donk (DeMott and Van Donk, 2013) noted that a moderately defended strain of *Oocystis* had a slower maximal growth rate than another green alga that showed no defenses (*Ankistrodesmus*). As expected, *Oocystis* dominated in grazer treatments, while *Ankistrodesmus* was more abundant in controls without grazers. However,

while results such as these are generally consistent with a defense-growth trade-off, convincing evidence for an evolutionary trade-off requires comparisons between closely related species or clones of a single species.

This study was stimulated by the preliminary observation that a gelatinous green alga isolated from a local lake improved in food quality for *Daphnia* during ~3 years in batch culture. In the original study, this alga (*Oocystis* B) supported the lowest *Daphnia* juvenile growth rate and the lowest assimilation efficiency (AE) of seven taxa of green algae tested (DeMott *et al.*, 2010). Juvenile growth rate for high food concentrations ranged 0.10 for *Oocystis* B to 0.61 day^{-1} for undefended algae. Juvenile growth was highly correlated with AE ($r^2 = 0.97$), a measure of digestibility, verifying the importance of digestion defenses for food quality. Thus, the digestion defenses of the *Oocystis* B isolate were initially very strong, but appeared to decline over time.

Here, we first sought to quantify the decline in grazer defenses in culture, using *Daphnia* juvenile growth as a proxy for algal defense. Evidence on the nature of the defense came from feeding experiments that tested whether the improved *Daphnia* growth was associated with improved digestibility, measured by carbon AE or an increase in feeding rate. We also made microscope observations and measurements to determine whether changes in defense were linked to changes in algal morphology, including cell and colony size and the incidence and diameter of the protective gelatinous sheath. Finally, we tested for a trade-off between grazer defense and the algal growth rate under nutrient replete conditions. We ran experiments with three *Oocystis* B sub-strains that were kept under differing conditions that strongly influenced the number of cell divisions since isolation. We hypothesized that culture lines undergoing a greater number of cell divisions would show greater evolutionary changes. We also made comparisons between the various *Oocystis* B sub-strains, a control high quality alga (*Ankistrodesmus*) and two strains of *Oocystis* from culture collections that had been isolated decades earlier.

METHOD

Organisms and culture methods

This study was stimulated by the observation that a gelatinous green alga (*Oocystis* B) was apparently losing its grazer defenses and improving in food quality for *Daphnia* during an extended period of laboratory culture. Here, we present data for four subcultures of *Oocystis* B, ranked by the relative number of generations since isolation. Strain 0 is represented by the original data on *Daphnia* juvenile growth and AE measured after 2–3 months in liquid culture (DeMott *et al.*, 2010). Between 3 and 3.5 years after the initial experiments, a series of experiments were run on three subcultures of *Oocystis* B that were kept under differing culture conditions that strongly influenced the relative number of generations since isolation. These included: an agar backup (sub-strain 1) that was refrigerated in the dark >90% of the time, a subculture kept at Indiana-Purdue University at Fort Wayne (IPFW, sub-strain 2) that was refrigerated ~75% of the time and a subculture that was continuously cultured in the light at Kellogg Biological Station (KBS, sub-strain 3). Thus, we were able to compare these three strains in common experiments using the same *Daphnia* clone and the same methods as the original study by DeMott *et al.* (DeMott *et al.*, 2010). *Oocystis* B and other gelatinous green algae were originally obtained from the guts of field-collected zooplankton using agar plates to isolate clones (DeMott *et al.*, 2010). We hypothesized that culturing clones in the absence of grazers would favor mutations that reduced investment in defense and that a greater number of generations in the absence of grazers would lead to weaker defenses. Since the KBS strain (strain 3) was actively growing about an order-of-magnitude longer than the agar backup (strain 1), we hypothesized the KBS strain would exhibit a greater decline in defenses than the agar backup strain and that the IPFW strain (strain 2) would be intermediate.

The experiments and comparisons included three taxa from culture collections (*Oocystis minuta*, UTEX LB 2071; *Oocystis lacustris*, SAG 81.80 and *Ankistrodesmus falcatus* as a high quality control). These *Oocystis* strains were maintained in the culture collections for >30 years before being used in experiments. Data on *Daphnia* growth and AE for *Oocystis* B, strain 0, *O. minuta* and *O. lacustris* were taken from DeMott *et al.* (DeMott *et al.*, 2010) while all data on *Daphnia* growth and assimilation for *Oocystis* strains 1, 2 and 3, and *Ankistrodesmus* and all algal growth rates are new. All experiments used cohorts of the same *Daphnia pulex* clone shown in previous experiments to be sensitive to variation in the digestibility of natural seston and cultured algae (DeMott and Tessier, 2002; DeMott *et al.*, 2010). This clone was raised in the laboratory on a diet of *Ankistrodesmus* for about one decade before the start of the experiments. Zooplankton were cultured in an artificial zooplankton medium (Tollrian, 1993) and algae were cultured in modified MBL medium (Stemberger, 1981) at room temperature (20–22°C) with continuous fluorescent light. All experiments were run with nutrient-rich algae from exponentially growing batch cultures using cohorts of *Daphnia* born within 24 h to mothers fed high concentrations of *Ankistrodesmus*. Algal biomass was estimated from absorbance at 750 nm,

using a spectrophotometer and calibration curves determined for each species and for each of the four *Oocystis* B sub-strains.

Zooplankton responses

Methods for estimating *Daphnia* juvenile growth (g, day^{-1}) and for using radioactively labeled (^{14}C-bicarbonate) algae to estimate clearance rate and AE are described in DeMott *et al.* (DeMott *et al.*, 2010). The *Daphnia* growth assays were started with neonates born during the previous 24 h and were run for 4 days with six to eight animals in separate beakers with 100 mL of medium and a high food concentration (1–2 mg L^{-1}). Food and medium were changed daily and animals were kept at 20°C under low light. At the end of each trial, the animals were dried and weighed individually to the nearest microgram. The growth rate g (day^{-1}) was calculated as the difference between the natural logarithm of the initial and final dry mass, divided by the experimental duration (4 days). Six juvenile growth experiments were conducted over a period of ~6 months, each with an *Ankistrodesmus* control. In total, we ran four replicate experiments for each of the three *Oocystis* B sub-strains. These new data were compared with original juvenile growth data for *Oocystis* B, sub-strain 0, *O. lacustris* and *O. minuta* from DeMott *et al.* (DeMott *et al.*, 2010).

Since younger, smaller animals have shorter gut passage times that make them more vulnerable to digestion defenses (DeMott *et al.*, 2010), a feeding and AE experiment was run with 2-day-old juveniles with the three *Oocystis* B sub-strains and *Ankistrodesmus*. There were four replicate beakers per algal strain with eight animals per beaker. A high food concentration (2 mg L^{-1}) ensured that food quantity was not limiting and that gut passage time was short. Animals were first acclimated with unlabeled algae and then ^{14}C-labeled algae was added at the same concentration. After feeding on the radioactively labeled algae for 5 min, four animals were immediately processed to estimate ingestion. The remaining four animals were transferred to unlabeled algae and allowed to clear their guts of radioactive food for 40 min. The gut passage time for small (2-day old) animals and high food concentration is <10 min (DeMott *et al.*, 2010). AE was estimated from the ratio of the long-term (after gut clearance):short-term (5 or 6 min) rates for animals feeding in each beaker.

Algal morphology and growth

Oocystis cells are typically surrounded by a diffuse gelatinous sheath that is not detected by electronic particle counters (personal observations) and is not visible under a microscope unless outlined by fine particles. The gelatinous sheath is thought to reduce digestibility, although a thick cell wall may also play a role (Porter, 1973; Van Donk *et al.*, 1997). We therefore placed *Oocystis* in suspensions of Indian ink and determined the number of cells per colony, the colony diameter, the incidence of sheaths and sheath diameter using a compound microscope at ×400. Measurements were made on two culture flasks for each of the three *Oocystis* subcultures and a minimum of 20 randomly selected colonies per flask. Replicates were measured ~1 month apart in order to include any uncontrolled variation in culture conditions. Observations on *O. minuta* and *O. lacustris* showed that these strains from culture collections lacked gelatinous sheathes and grew exclusively as single cells.

Algal growth rates were measured by placing dilute 9 mL cultures in 50 mL flasks on a shaker table under continuous fluorescent light (80 μE m^{-2} s^{-1}) with three replicate flasks per algal strain. The shaker table rotated 1 min of every 10 min. Algal volume was measured initially (Day 0) and every 24 h for 5 days using a Coulter Z2 electronic particle counter. The growth rate r (day^{-1}) was estimated from the slope of a regression of the natural log of volume versus days. Data for Days 1–4 or 1–5 were used, since these data gave the best regression fits ($r^2 > 0.98$). The *Oocystis* B sub-strain 2 culture was lost before we conducted the algal growth experiments. This sub-strain could not be included in the algal growth versus grazer defense trade-off analysis.

Experiments on feeding, algal growth rates and comparisons of algal morphology used beakers or flasks as replicates. Data on *Daphnia* growth summarize experiments run months or years apart using the same experimental methods. In this case, the overall mean for an experiment was considered one replicate and statistical analysis was conservatively based on variation between replicate experiments run on different dates. All experiments were analyzed by one-way ANOVA with the Tukey method for testing *a posteriori* contrasts.

RESULTS

Zooplankton responses

Daphnia juvenile growth assays show that the various strains of nutrient-sufficient *Oocystis* represent a very wide gradient in food quality (Fig. 1, ANOVA, $F = 38.0$, $P < 0.001$). At one extreme, *O. lacustris* and *O. minuta*, which were isolated >30 years earlier, supported maximal growth rates comparable to the high quality control, *Ankistrodesmus*. Different sub-strains of *Oocystis* B also represent a wide gradient in food quality, ranging

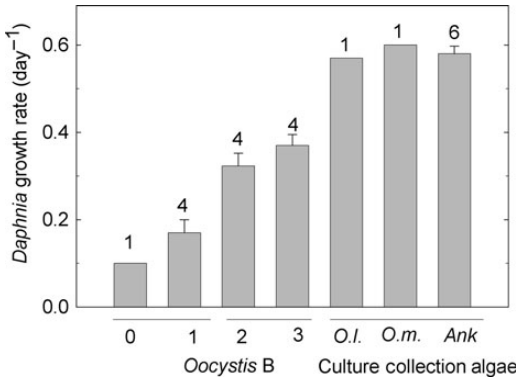

Fig. 1. Comparison of *Daphnia* juvenile growth rate for four sub-strains of *Oocystis* B, *Oocystis* from culture collections (*O. l.*, *O. lacustris; O.m.*, *O. minuta*) and the high quality control, *Ankistrodesmus* (*Ank*). Data for sub-strain 0, *O. lacustris* and *O. minuta* were taken from DeMott *et al.* (DeMott *et al.*, 2010). Experiments with sub-strain 0 were run within 3 months of isolation into liquid culture. Experiments with sub-strains 1, 2 and 3 and *Ankistrodesmus* were run after 3 years of culture. Sub-strain 3 was cultured continuously in the light, sub-strain 1 was grown in the light <10% of the time while sub-strain 2 was grown in the light ~25% of the time. Data are mean ± SE for replicate experiments (numbers above bars). Treatments not connected by lines are significantly different ($P < 0.05$).

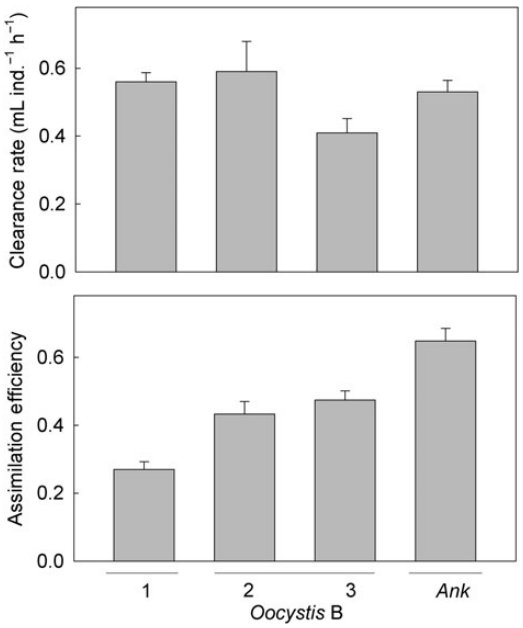

Fig. 2. Comparison of clearance rates (above) and assimilation efficiencies (below) for 2-day-old *Daphnia* fed high concentrations (2 mg/L) of three sub-strains of *Oocystis* B and the high quality control alga, *Ankistrodesmus*. See Fig. 1 for explanation of the sub-strains. Data are mean ± SE for four replicates. Treatments not connected by lines are significantly different ($P < 0.05$).

from algae used in the first experiments soon after isolation (sub-strain 0, $g = 0.10\ \text{day}^{-1}$) to the sub-strain grown continuously in the light for ~3 years (sub-strain 3, $0.38\ \text{day}^{-1}$). Comparisons among *Oocystis* B sub-strains 1, 2 and 3 support our hypothesis that the improvement in food quality in culture is related to the relative number of generations since isolation.

A feeding experiment with 2-day-old *Daphnia* tested whether the gradient in food quality was due to differences in AE or other factors, such as variation in feeding rate. In support of the digestibility hypothesis, AE was lowest for *Oocystis* B sub-strain 1, intermediate for sub-strains 2 and 3 and highest for *Ankistrodesmus* (Fig. 2, lower panel, ANOVA, $F = 23.8$, $P < 0.001$). On the other hand, clearance rates did not differ among the four algal treatments (Fig. 2, upper panel, ANOVA, $F = 2.38$, $P = 0.12$). Figure 3 shows a linear relationship between AE and *Daphnia* juvenile growth rate for all four sub-strains of *Oocystis* B and the three culture collection algae (linear regression, $n = 7$, $r^2 = 0.90$, $F = 56.7$, $P = 0.001$).

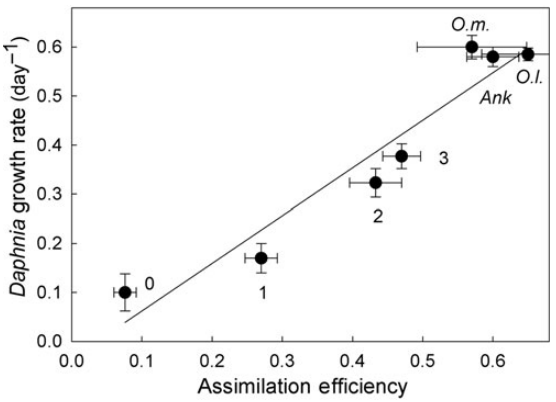

Fig. 3. Relationship between AE of 2-day-old *Daphnia* and *Daphnia* juvenile growth for four sub-strains of *Oocystis* B and three algae from culture collections. See Fig. 1 for explanation of the sub-strains and symbols. Symbols show mean ± SE. Line is a least squares regression. Data for *Oocystis* B sub-strain 0 and *O. l.* and *O. m.* are from DeMott *et al.* (DeMott *et al.*, 2010).

Algal morphology

Because we did not measure the algal morphology (or growth rate) of the newly isolated *Oocystis* B (sub-strain 0), our microscope measurements are limited to *Oocystis* B strains 1, 2 and 3. The three sub-strains were similar in cell size, but differed in the incidence of colonies, the incidence of gelatinous sheaths and in sheath diameter (Table I). *Oocystis* typically grows as single cells and as colonies of four cells contained within the old mother cell wall. All three sub-strains showed a modal colony size of four cells. The incidence of colonies increased with increasing time of active growth, with strain 1 lowest, strain 3 highest and strain 2 intermediate. In contrast, both the

Table I: Morphological characteristics of Oocystis *B sub-strains*

	Strain 1	Strain 2	Strain 3	*F*	*P*
Cell diameter (μm)	8.9 ± 0.45	8.8 ± 0.25	9.8 ± 0.25	2.78	0.21
Colonies					
Incidence	0.19 ± 0.06	0.32 ± 0.09	0.63 ± 0.15	11.7	0.038
Cells/colony	3.9 ± 0.10	3.6 ± 0.10	3.9 ± 0.24	1.44	0.37
Sheath					
Incidence	1.0 ± 0.00	0.93 ± 0.03	0.59 ± 0.03	111	0.002
Diameter (μm)	93 ± 10	71 ± 5	36 ± 3	21.8	0.016

The three *Oocystis* B sub-strains were isolated ~3 years before the microscope measurements and were kept under conditions ranging from >95% of time in dark (sub-strain 1) to continuous light (sub-strain 3). Data show means ± SE for measurements made from two culture flasks. Treatments not connected by a line are significantly different ($P < 0.05$).

incidence of sheaths and sheath diameter declined with time of active growth. Notably, all of the *Oocystis* sub-strain 1 cells and colonies that we observed produced a gelatinous sheath, whereas only ~60% of the sub-strain 3 cells and colonies produced sheaths. The sheath diameter for sub-strain 1 was nearly three times greater than sub-strain 3, while sub-strain 2 was intermediate but was more similar to sub-strain 1. The two culture collection strains of *Oocystis* both grew as single cells lacking sheaths. Thus, the culture collection *Oocystis* are consistent with the observation that the *Oocystis* gradually reduces mucilage production in culture. On the other hand, the observed increase in the incidence of colonies over culture time in *Oocystis* B is inconsistent with the lack of colonies in the culture collection algae.

Algal growth rate versus defense trade-off

Algal exponential growth rates were measured in nutrient-rich medium and with continuous, moderately high light intensity. The growth rate was lowest for *Oocystis* B, strain 1, highest for three culture collection algae and intermediate for *Oocystis* B, strain 3 (ANOVA, $F = 27.7$, $P < 0.001$). Algal growth rate was lowest for the strain with the strongest digestion defenses (*Oocystis* B, strain 1), highest for the three undefended algae and intermediate for *Oocystis* B, strain 3. Thus, a plot algal growth rate versus *Daphnia* growth rate provides support for an algal growth rate versus grazer defense trade-off (Fig. 4, linear regression, $n = 5$, $r^2 = 0.92$, $F = 47.7$, $P = 0.006$).

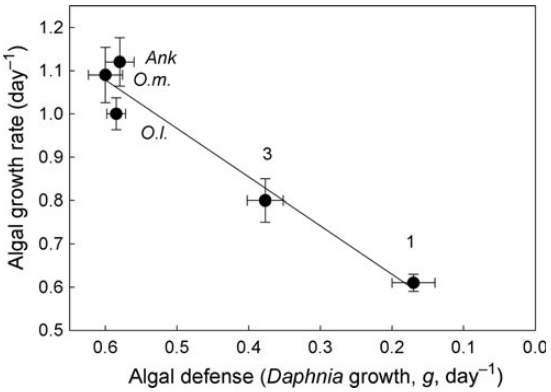

Fig. 4. Trade-off between algal growth rate and algal defense for two sub-strains of *Oocystis* B (1 and 3), two strains of *Oocystis* from culture collections (*O. l.* and *O. m.*) and the high quality control *Ankistrodesmus*. *Daphnia* growth is a proxy for algal defense, where reduced *Daphnia* growth indicates a stronger defense. Symbols show mean ± SE. Line is a least squares regression.

DISCUSSION

Loss of grazer defenses in laboratory cultures

In this study, *Daphnia* juvenile growth at high food concentrations was used as a proxy for algal defenses. In order to quantify a decline in algal defenses over a period of over 3 years, we used a single clone of *Daphnia* and the same methods for assessing *Daphnia* juvenile growth and AE. The *Daphnia* clone was maintained in the laboratory for over a decade on a diet of high quality *Ankistrodesmus* before our experiments. Thus, it is unlikely that the *Daphnia* clone evolved a greater ability to overcome digestion defenses during the 3-year period of this study. Similar results for the high quality control alga (*Ankistrodesmus*) support comparisons between the earlier (DeMott *et al.*, 2010) and current study (mean ± SE; juvenile growth 0.58 ± 0.02 versus 0.60 ± 0.01 day^{-1}; AE for 2-day old juveniles 0.57 ± 0.06 versus 0.64 ± 0.04).

Our experiments demonstrate an improvement in *Daphnia* growth on strains of a gelatinous green alga over several years of laboratory culture. Most importantly, contemporary comparisons between *Oocystis* strains 1, 2 and 3 support our hypothesis that that the loss of defenses

depends on the relative number of algal generations in culture. In agreement with earlier studies on gelatinous green algae (e.g. Porter, 1975; Vanni and Lampert, 1992; DeMott *et al.*, 2010), the digestion defense hypothesis was supported by feeding studies showing that AE, a measure of digestibility, accounted for most of the variation in the juvenile growth rate among sub-strains of *Oocystis* B and the *Oocystis* B strains and three undefended algae. On the other hand, clearance and ingestion rates did not differ between the three *Oocystis* strains and *Ankistrodesmus*, suggesting that differences in feeding rates did not account for the differences in *Daphnia* juvenile growth.

Studies on marine copepods provide evidence for very high AE of elements associated with the cytoplasmic fraction of algae and very low AE for elements associated with cell walls (Reinfelder and Fisher, 1991). We speculate that herbivorous cladocerans use a similar digestive mechanism and that once the defenses of a digestion-resistant alga are breached, the cytoplasmic fraction is rapidly assimilated. In this scenario, differences in AE between strongly defended, weakly defended and undefended green algae would reflect the proportion of cells surviving gut passage. Thus, our results quantify the loss of grazer defenses and help verify that digestion resistance is the mechanism of defense. Since both *Daphnia* juvenile growth rate and AE remained below that of the three undefended green algae, including two species of *Oocystis*, we conclude that even the strain of *Oocystis* B cultured in continuous light retained substantial grazer defenses.

Direct comparisons among the three sub-strains of *Oocystis* B provide evidence that the loss of grazer defenses was a gradual process, dependent on the number of cell divisions over several years. This pattern of evolutionary change is consistent with a quantitative genetic trait determined by mutations at many gene loci, each with a small, incremental effect (Houle, 1992; Lakeman *et al.*, 2009)

Previous evidence for the role of gelatinous sheaths in viable gut passage comes largely from comparisons between phytoplankton species with and without protective sheaths (e.g. Porter, 1973, 1975; Vanni and Lampert, 1992; DeMott *et al.*, 2010). Our study provides more direct evidence about the role of the sheath as a defense. This study is the first to suggest that variation in sheath thickness plays a role in the strength of the grazer defense. Our results indicate that the mucilage production and sheath formation are not an all or nothing trait but provide a stronger defense with increasing diameter. However, *Oocystis* strain 2 was more similar to strain 1 in sheath diameter, but more similar to strain 3 in AE and *Daphnia* growth. Thus, understanding digestion defenses is probably more complex than just measuring sheath diameter. Measuring the relationship between sheath thickness and zooplankton growth in a variety of clones of gelatinous algae isolated from lakes could provide more realistic evidence on the role of sheath thickness in grazer defenses. Many algae have gelatinous sheaths that are readily visible under a microscope and appear much more robust than the diffuse sheaths of *Oocystis* (DeMott, 1995; Reynolds, 2007). However, there is no direct evidence on the relative roles of sheath thickness and consistency in digestion defenses.

Since the gelatinous sheath of *Oocystis* dramatically increases colony diameter, the sheath could protect against ingestion as well as digestion. However, in agreement with earlier studies of *Daphnia* and *Oocystis* (Vanni and Lampert, 1992; DeMott *et al.*, 2010), our feeding experiment confirms that the large but diffuse gelatinous sheath of *Oocystis* is surprisingly ineffective in protecting against ingestion. This result is generally consistent with the finding that soft algae including gelatinous greens are much more readily ingested than plastic beads or hard algae (e.g. diatoms and dinoflagellates; DeMott, 1995). Furthermore, large colonies of the gelatinous green *Eurdorina* from a laboratory culture with softer sheaths were more readily ingested than *Eudorina* from a field population with a more robust sheath (DeMott, 1995). Unlike *Oocystis*, the gelatinous sheath of *Eurdorina* is readily visible under a microscope. We hypothesize that the sheathes of *Oocystis* and other gelatinous greens are deformed to varying degrees during ingestion. Unfortunately, we have no means of quantifying sheath properties other than diameter that may influence ingestion or digestion.

According to Reynolds (Reynolds, 2007), phytoplankton often reduce or cease mucilage production in culture. For example, the cyanobacterium *Microcystis* forms colonies embedded in a mucilaginous matrix in nature but typically grows as single cells in laboratory culture. Interestingly, field-isolated clones differ in growth rate, colony size and the rate at which the colonial morphology is lost in culture (Wilson *et al.*, 2006). The gelatinous green alga *Sphaerocystis* has a colony structure similar to *Microcystis* in nature, but strains from culture collections also grow as single cells (W.R. DeMott, personal observation). The lack of gelatinous sheaths in the two *Oocystis* species from culture collections is indirect evidence that such sheaths are eventually lost during culture. While algae with protective gelatinous sheaths seem to support the use it or lose it paradigm, there is little evidence that other mechanisms of grazer defense deteriorate with time in culture. Studies of digestion defenses of *Chlorella* against rotifer grazing provide an interesting counterpoint to *Oocystis*. Although Meyer *et al.* (Meyer *et al.*, 2006) provide convincing evidence of viable gut passage, *Chlorella* lacks a sheath and the mechanism of digestion resistance is unclear, despite unpublished studies on cell wall morphology by Yoshida *et al.* (Yoshida *et al.*, 2004). In these *Chlorella* examples, substantial variation in

digestion defenses was found within and between culture collection strains. Thus, the grazer defenses apparently both evolved and were maintained in laboratory cultures for more than 50 years in the absence of grazers.

Grazer defense versus growth trade-off

Our study is one of a few that provide direct evidence for the evolution of a growth rate versus grazer defense trade-off in phytoplankton. We studied the effects of relaxing predation, rather than increasing predation or changing the type of predator. The most important evidence comes from comparisons in growth and defense between a strain kept in the dark most of the time (sub-strain 1) and a strain growth continuously in the light (sub-strain 3). As predicted, a greater relative number of algal generations in the absence of predation lead to increased algal growth rate and a greater loss of defenses. Unfortunately, we did not test the growth rate of *Oocystis* B soon after isolation (strain 0), although our impression was that growth was very slow (W.R. DeMott, personal observation). Some algae showed reduced maximal growth after long-term culture (Sweeney, 1986; Berge *et al.*, 2012). Thus, batch culture conditions do not necessarily select for increased algal growth rates.

Since we only tested algal growth under nutrient replete conditions, we do not have evidence on whether less investment in defenses also leads to better competitive ability when nutrients are limiting. Meyer *et al.* (Meyer *et al.*, 2006) found that digestion-resistant *Chlorella* had lower growth rates over a range of limiting nitrogen concentrations in comparison with an undefended strain. They proposed that the defenses that protect against digestive enzymes also reduce nutrient uptake, leading to reduced growth over both high and low nutrient levels. Consistent with this hypothesis, Sunda and Hardison (Sunda and Hardison, 2010) showed that poorly grazed species and strains of marine phytoplankton had both lower rates of ammonium uptake and lower growth rates over a gradient in ammonium concentrations. In contrast, Yoshida *et al.* (Yoshida *et al.*, 2004) found that a poorly defended strain of *Chlorella* had a higher growth rate only under low, limiting nitrogen concentrations. It seems very plausible that digestion defenses reduce growth by slowing nutrient uptake, but this hypothesis needs further testing.

Reviews show that algae with grazer defenses are often most successful when grazing is strong, a finding consistent with a defense versus growth trade-off (Sterner, 1989; Agrawal, 1998). However, the same reviews point to exceptions where poorly defended algae dominate under strong grazing (also see Sarnelle, 2005) or where increased grazing causes the loss of defended species (also see Tessier *et al.*, 2001). A number of complexities that affect the strength of digestion defenses or alter the importance of growth rate differences may explain the exceptions. Digestion defenses are more effective when seston concentration is high and gut passage time is short (DeMott *et al.*, 2010). High algal concentrations are typically associated with low dissolved nutrients, a condition which should reduce the importance of differences in maximal growth rate. Furthermore, DeMott and Van Donk (DeMott and Van Donk, 2013) showed that phosphorus-limited conditions strengthened digestive defenses, while Porter (Porter, 1976) showed that gelatinous algae can take up phosphorus from grazer guts during viable gut passage. Thus, these two mechanisms should enhance the advantage of digestion defenses under algal nutrient limitation, a condition not tested in this study. On the other hand, when algal concentrations are low and nutrients and light are high, the advantage may shift to fast-growing undefended species and strains (Grover and Holt, 1998; Sarnelle, 2005)

Understanding trade-offs that operate between and within species is a key to understanding the interplay between phytoplankton community structure and evolution (Litchman *et al.*, 2012). While studies of evolution in laboratory cultures show the potential for a defense versus growth trade-off, we clearly need studies that test for defense versus growth trade-offs between field-collected clones. Our study cautions that the potential for loss of defenses needs to be considered in laboratory food chain experiments.

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REFERENCES

Agrawal, A. A. (1998) Algal defenses, grazers and their interactions in aquatic trophic cascades. *Acta Oecol.*, **19**, 331–337.

Becks, L., Ellner, S. P., Jones, L. E. *et al.* (2010) Reduction of genetic diversity radically alters eco-evolutionary community dynamics. *Ecol. Lett.*, **13**, 989–997.

Berge, T., Daugbjerg, N. and Hansen, P. J. (2012) Isolation and cultivation of microalgae select for low growth rate and tolerance to high pH. *Harmful Algae*, **20**, 101–110.

Brand, L. E. (1981) Genetic variability in reproduction rates in marine phytoplankton populations. *Evolution*, **35**, 117–1127.

Brand, L. E. (1989) Review of genetic variation in marine phytoplankton species and the ecological implications. *Biol. Oceanogr.*, **6**, 397–409.

Costas, E., Lopez-Rodas, V., Salgado, C. *et al.* (1998) Adaptation to competition by new mutation in clones of *Alexandrium minutum*. *Evolution*, **52**, 610–613.

DeMott, W. R. (1989) The role of competition in zooplankton succession. In Sommer, U. (ed.), *Plankton Ecology: Succession in Plankton Communities*. Springer-Verlag, Berlin, pp. 195–253.

DeMott, W. R. (1995) The influence of prey hardness on *Daphnia*'s selectivity for large prey. *Hydrobiologia*, **307**, 127–138.

DeMott, W. R., McKinney, E. N. and Tessier, A. J. (2010) Ontogeny of digestion in *Daphnia*: implications for the effectiveness of algal defenses. *Ecology*, **91**, 540–548.

DeMott, W. R. and Tessier, A. J. (2002) Stoichiometric constraints vs. algal defenses: testing mechanisms of zooplankton food limitation. *Ecology*, **83**, 3426–3433.

DeMott, W. R. and Van Donk, E. (2013) Strong interactions between stoichiometric constraints and algal defenses: evidence from population dynamics of *Daphnia* and algae in phosphorus-limited microcosms. *Oecologia*, **171**, 175–186.

Edwards, K. R., Thomas, M. K., Klausmeier, C. A. *et al.* (2012) Allometric scaling and taxonomic variation in nutrient utilization traits and maximum growth rate of phytoplankton. *Limnol. Oceanogr.*, **57**, 554–556.

Grover, J. P. and Holt, R. D. (1998) Disentangling resource and apparent competition: realistic models for plant-herbivore communities. *J. Theor. Biol.*, **191**, 353–376.

Houle, D. (1992) Comparing evolvability and variability of quantitative traits. *Genetics*, **130**, 195–204.

Kaebernick, M., Rohrlack, T., Christoffersen, K. *et al.* (2001) A spontaneous mutant of microcystin biosynthesis: genetic characterization and effect on *Daphnia*. *Environ. Microbiol.*, **3**, 669–679.

Kampe, H., König-Rinke, M., Petzoldt, T. *et al.* (2007) Direct effects of *Daphnia*-grazing, not infochemicals, mediate a shift towards large inedible colonies of the gelatinous green alga *Sphaerocystis schroeteri*. *Limnologica*, **37**, 137–145.

Kerfoot, W. C., Levitan, C. and DeMott, W. R. (1988) *Daphnia*-phytoplankton interactions: density dependent shifts in resource quality. *Ecology*, **69**, 1806–1825.

Lakeman, M. B. and Cattolico, R. A. (2007) Cryptic diversity in phytoplankton cultures is revealed using a simple plating technique. *J. Phycol.*, **43**, 662–674.

Lakeman, M. B., von Dassow, P. and Cattolico, R. A. (2009) The strain concept in phytoplankton ecology. *Harmful Algae*, **8**, 746–758.

Litchman, E., Edwards, K. F., Klausmeier, C. A. *et al.* (2012) Phytoplankton niches, traits and eco-evolutionary responses to global environmental change. *Mar. Ecol. Prog. Ser.*, **470**, 235–248.

Lynch, M., Gabriel, W. and Wood, A. M. (1991) Adaptive and demographic responses of plankton populations to environmental change. *Limnol. Oceanogr.*, **36**, 1301–1312.

Martins, C. A., Kulis, D., Franca, S. *et al.* (2004) The loss of PSP toxin production in a formerly toxic *Alexandrium lusitanicum* clone. *Toxicon*, **43**, 195–205.

Meyer, J. R., Ellner, S. P., Hairston, N. G. Jr *et al.* (2006) Prey evolution on the time scale of predator-prey dynamics revealed by allele-specific quantitative PCR. *Proc. Natl Acad. Sci. U.S.A.*, **103**, 10690–10695.

Porter, K. G. (1973) Selective grazing and differential digestion of algae by zooplankton. *Nature*, **244**, 179–180.

Porter, K. G. (1975) Viable gut passage of gelatinous green algae ingested by *Daphnia*. *Int. Ver. Theor. Angew. Limnol. Verh.*, **19**, 2840–2850.

Porter, K. G. (1976) Enhancement of algal growth and productivity by grazing zooplankton. *Science*, **192**, 1332–1334.

Reinfelder, J. R. and Fisher, N. S. (1991) The assimilation of elements by marine copepods. *Science*, **251**, 794–796.

Reynolds, C. S. (2007) Variability in the provision and function of mucilage in phytoplankton: facilitative responses to the environment. *Hydrobiologia*, **578**, 37–45.

Rynearson, T. A. and Armbrust, E. V. (2000) DNA fingerprinting reveals extensive genetic diversity in a field population of the centric diatom *Ditylum brightwellii*. Limnol. Oceanogr., **45**, 1329–1340.

Sarnelle, O. (2005) *Daphnia* as keystone predators: effects on phytoplankton diversity and grazing resistance. *J. Plankton Res.*, **27**, 1227–1238.

Schatz, D., Keren, Y., Hadas, O. *et al.* (2005) Ecological implications of the emergence of non-toxic subcultures from toxic *Microcystis* strains. *Environ. Microbiol.*, **7**, 798–805.

Stemberger, R. (1981) A general approach to the culture of rotifers. *Can. J. Fish Aquat. Sci.*, **38**, 721–724.

Sterner, R. W. (1989) The role of grazers in phytoplankton succession. In Sommer, U. (ed.), *Plankton Ecology: Succession in Plankton Communities*. Springer-Verlag, Berlin, pp. 107–170.

Sunda, W. G. and Hardison, D. R. (2010) Evolutionary tradeoffs among nutrient acquisition, cell size, and grazing defense in marine phytoplankton promote ecosystem stability. Mar. Ecol. Prog. Ser., **401**, 63–76.

Sweeney, B. M. (1986) The loss of circadian rhythm in photosynthesis in an old strain of *Gonyaulax polyedra*. *Plant Physiol.*, **80**, 978–981.

Tessier, A. J., Bizina, E. V. and Geedey, C. K. (2001) Grazer-resource interactions in the plankton: Are all daphniids alike? *Limnol. Oceanogr.*, **46**, 1585–1595.

Tessier, A. J. and Woodruff, P. (2002) Cryptic trophic cascade along a gradient of lake size. *Ecology*, **83**, 1263–1270.

Tollrian, R. (1993) Neckteeth formation in *Daphnia pulex* as an example of continuous phenotypic plasticity: morphological effects of *Chaoborus* kairomone concentration and their quantification. *J. Plankton Res.*, **15**, 1309–1318.

Van Donk, E., Lürling, M., Hessen, D. O. *et al.* (1997) Altered cell wall morphology in nutrient-deficient phytoplankton and its impact on grazers. *Limnol. Oceanogr.*, **42**, 357–364.

Vanni, M. J. (1987) Effects of nutrients and zooplankton size on the structure of a phytoplankton community. *Ecology*, **68**, 624–635.

Vanni, M. J. and Lampert, W. (1992) Food quality effects on life history traits and fitness in the generalist herbivore *Daphnia*. *Oecologia*, **92**, 48–57.

Vanormelingen, P., Vyverman, W., De Bock, D. *et al.* (2009) Local genetic adaptation to grazing pressure of the green alga *Demodesmus armatus* in a strongly connected pond system. *Limnol. Oceanogr.*, **54**, 503–511.

White, J. D., Kaul, R., Knoll, L. B. *et al.* (2011) Large variation in vulnerability to grazing within a population of the colonial phytoplankter *Microcystis aeruginosa*. *Limnol. Oceanogr.*, **56**, 1714–1724.

Wilson, A. E., Wilson, W. A. and Hay, M. E. (2006) Intraspecific variation in growth and morphology of the bloom-forming cyanobacterium *Microcystis aeruginosa*. Appl. Environ. Microbiol., **72**, 7386–7389.

Yoshida, T., Hairston, N. G. Jr and Ellner, S. P. (2004) Evolutionary trade-off between defense against grazing and competitive ability in a simple unicellular alga, *Chlorella vulgaris*. *Proc. R. Soc. Lond. Ser. B*, **271**, 1947–1953.

Yoshida, T., Jones, L. E., Ellner, S. P. *et al*. (2003) Rapid evolution drives ecological dynamics in a predator-prey system. *Nature*, **424**, 303–306.